Obesity in BSB Mice Is Correlated with Expression of Genes for Iron Homeostasis and Leptin

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Abstract

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Objective: We searched for genes whose alleles cause obesity and novel pathways correlated with obesity.

Research Methods and Procedures: BSB mice are a model of complex obesity due to interactions among genes from C57BL/6J (B) and *Mus spretus* (SPRET) in (B × SPRET) × B backcross mice. Stringent criteria identified 50 genes differentially expressed in epididymal adipose tissue from 7 pairs of lean vs. obese BSB mice. Quantitative reverse transcription-polymerase chain reaction of adipose tissue RNA from 48 BSB mice with a range of obesity was assayed. Leptin was evaluated in inbred (SPRET/Ei) and outbred (SPRET/Pt) BSB mice.

Results: Leptin (*Lep*) and adipsin expressions had the greatest fold differences between obese and lean mice. Four genes involved in iron homeostasis were included in the 50 differentially expressed genes [hemochromatosis (*Hfe*), diaphorase 1, transferrin receptor (*Trfr*) 2, and protoporphyrinogen oxidase] and two additional iron-related genes did not quite meet the stringent criteria for differential expression (*Trfr* and lactotransferrin). *Hfe* and *Trfr* mRNA levels and liver iron were negatively correlated with fat mass. Variation in obesity phenotypes explained 49%, 40%, and 37%, respectively, of the variance in *Hfe*, *Lep*, and *Trfr* mRNA levels. Leptin differed by haplotype at the *Lep* locus in outbred BSB. The quantitative trait locus identified in the outbred cross did not occur in inbred BSB.

Discussion: Our results suggest that iron homeostasis in BSB mice is coordinately regulated in vivo in adipose depots in response to obesity. *Lep* alleles derived from outbred, but not inbred, SPRET are a positional candidate for the chromosome 6 quantitative trait locus in BSB mice.

Key words: expression profiling, iron homeostasis, hemochromatosis, leptin, liver iron

Introduction

Obesity is a complex disease. To date, over 100 chromosomal loci for body weight, body fat, regional white adipose tissue weight, and other obesity-related traits have been identified in humans and in animal models (1). For most loci, the underlying genes are not yet identified; some of these chromosomal loci will be alleles of known obesity genes, whereas many will represent alleles of unknown genes.

Microarray analysis allows simultaneous multiple gene and pathway discovery. cDNA and oligonucleotide arrays are commonly used to identify differentially expressed genes by surveys of large numbers of known and unnamed genes (2,3). Two papers previously identified genes differentially expressed in adipose tissue of mouse models of obesity and diabetes by analysis of hybridization to Affymetrix oligonucleotide chips (4,5). A paper by Nadler et al. (4) compared the effect of obesity without diabetes,

resulting from the *ob* mutation on the C57BL/6J (B)[1] background, with obesity and overt diabetes, resulting from the *ob* mutation on the BTBR mouse background, on gene expression in white adipose tissue. Obesity in this model was associated with decreased expression of genes important in adipocyte differentiation, and diabetes also was associated with changes in genes of signal transduction and energy metabolism. The paper by Soukas et al. (5) examined the response of gene expression in white adipose tissue to different ambient levels of leptin and to food restriction, again using the *ob* model, and observed down-regulation of genes involved in fatty acid biosynthesis and up-regulation of genes involved in inflammation. Both studies found that leptin is up-regulated by obesity. Moraes et al. (6) examined gene expression in adipose tissue in a multigenic model of obesity induced by a high-fat diet in B male mice. They also found that genes involved in lipid metabolism and adipocyte differentiation were down-regulated and those involved in inflammation were up-regulated in obese mice.

Obesity in BSB mice results from the interaction of multiple genes (7) and may not have the same underlying causes as monogenic obesity models. BSB mice, derived from backcrossing B to F1 [B × *Mus spretus* (SPRET)], are a model of spontaneous multigenic obesity (8). Although parental strains and F1s remain lean on low-fat diets, BSB mice display a wide range of adiposity-related phenotypes: fat as percentage of body weight ranges from <1% to >50% in low-fat-fed BSB mice. Thus, the underlying causes of obesity in BSB mice are inherently complex. Quantitative trait locus (QTL) mapping using outbred SPRET/Pt previously identified four QTL regions for percentage body fat or the weight of individual fat depots on chromosomes 6, 7, 12, and 15 (7,9), in addition to a later-reported chromosome 2 QTL (10). The mouse leptin gene is near the peak of the chromosome 6 QTL.

SPRET mice that are either inbred (SPRET/Ei) or outbred (SPRET/Pt) have been used to produce BSB mice. Studies by other investigators demonstrate that outbred SPRET can have haplotypes that may promote epidermal cancer or that have no effect, whereas inbred SPRET/Ei may or may not share these haplotypes (11). Haplotype analysis of inbred and outbred SPRET was used to identify a 1- to 2-centiMorgan (cM) interval containing the epidermal cancer-promoting gene (11). Because BSB mice are produced by breeding F1 females to B6 males, effects of different haplotypes can be detected by searching for differences among the progeny of different F1 mothers.

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[1] Nonstandard abbreviations: B, C57BL/6J; SPRET, *Mus spretus*; QTL, quantitative trait locus; cM, centiMorgan; *Hfe*, hemochromatosis; *Lipc*, hepatic lipase; AI, adiposity index; RT, reverse transcription; EST, expressed sequence tag; PCR, polymerase chain reaction; SSC, standard sodium citrate; qRT, quantitative real-time; Ct, threshold cycle; *Lep*, Leptin; *Adn*, adipsin; *Trfr*, transferrin receptor; *Cyp2e1*, cytochrome P450 2e1; *Ptplb*, protein tyrosine phosphatase-like B; *Mmp11*, matrix metalloproteinase-11.

In the present study, gene expression profiles of lean and obese BSB epididymal adipose tissue RNA are compared. We identified a total of 50 genes as differentially expressed 2-fold or more and with *p* values of ≤0.001 for comparisons between the leanest and the most obese mice. We followed up these observations with more detailed studies of the role of the hemochromatosis (*Hfe*) gene and iron homeostasis in BSB obesity and with tests of the role of leptin alleles in BSB obesity.
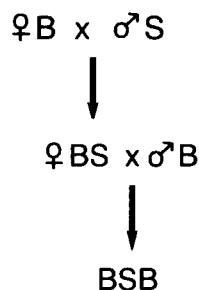
Research Methods and Procedures

Mice

Parental SPRET (SPRET/Pt and SPRET/Ei), *Mus musculus domesticus* (B), and mice with disrupted hepatic lipase ($Lipc^{-/-}$) alleles bred on the B background were obtained from The Jackson Laboratory (Bar Harbor, ME) or from the laboratory of Mike Potter (SPRET/Pt; NIH, Bethesda, MD) as described previously (8). Breeding schemes for making outbred (using SPRET/Pt) or inbred (using SPRET/Ei) SPRET BSB mice are shown in Figure 1. The *Lipc* knockout on the B background was used to generate BSB mice with different combinations of alleles at *Lipc* as part of a study on the effect of *Lipc* on obesity that appears in this issue of *Obesity Research* (12). The *Lipc* alleles should not affect expression results as long as the genes of interest do not interact with *Lipc*.

After weaning at 21 d of age, male BSB mice were housed individually in plastic shoebox cages and had ad libitum access to water and a standard chow diet (Purina Rodent Chow 5001; Research Diets, New Brunswick, NJ) containing 12% of energy as fat. Room temperature was kept at 20 to 22 °C with alternate 14-h-light and 10-h-dark cycles. Adult ~5-month-old BSB mice were killed after an overnight fast. Before sacrifice, mice were anesthetized with inhaled isoflurane and were bled through the retroorbital sinus. The epididymal fat pad was dissected, weighed, and immediately frozen in liquid nitrogen and stored at −80 °C. Three fat pads, consisting of two intra-abdominal (mesenteric and bilateral retroperitoneal) fat pads and a subcutaneous fat pad from the outer thigh (bilateral femoral), were dissected, weighed, and replaced for establishing percentage body fat. Body fat was corrected for the estimated fat in the epididymal fat pad. Body composition, using the Soxhlet apparatus, was used to determine percentage body fat of the mice (13). Total fat pad was defined as the summed weight of the four dissected adipose depots. BMI was calculated by dividing live body weight by the square of nasal-anal length (grams per centimeter squared), and adiposity index (AI) was calculated as total fat pad weight divided by total carcass weight.

All animals were housed and cared for under conditions meeting the NIH standards as stated in the *Guide for the Care and Use of Laboratory Animals*, American Association for Accreditation of Laboratory Animal Care accreditation standards, and the Animal Welfare Act PL85-544.

Wildtype Cross: C57BL/6J (B) x SPRET/Pt (S)

♀B x ♂S

↓

♀BS x ♂B

↓

BSB

Lipc$^{-/-}$ Cross: C57BL/6J (B) x SPRET/Ei (S)

Cross #1:

♀B (*Lipc*$^{-/-}$) x ♂S (*Lipc*^{SS})

↓

♀BS (*Lipc*$^{S-}$) x ♂B (*Lipc*$^{-/-}$)

↓

BSB (*Lipc*$^{S-}$)
BSB (*Lipc*$^{-/-}$)

Cross #2:

♀B (*Lipc*$^{-/-}$) x ♂S (*Lipc*^{SS})

↓

♀BS (*Lipc*$^{S-}$) x ♂B (*Lipc*^{BB})

↓

BSB (*Lipc*^{BS})
BSB (*Lipc*$^{B-}$)

Cross #3

♀B (*Lipc*^{BB}) x ♂S (*Lipc*^{SS})

↓

♀BS (*Lipc*^{BS}) x ♂B (*Lipc*$^{-/-}$)

↓

BSB (*Lipc*$^{B-}$)
BSB (*Lipc*$^{S-}$)

Figure 1: Breeding schemes for BSB mice. Four separate crosses were used: first, a cross using outbred SPRET/Pt mice and wild-type B mice; then, crosses using inbred SPRET/Ei mice and various combinations of wild-type B and *Lipc*$^{-/-}$ knockout mice on the B background.

cDNA Microarrays

Epididymal adipose total RNA from mice in the highest and the lowest adiposity extremes from crosses 1 and 2 as measured by percentage body fat (Figure 2A; Table 1) was isolated using TRIzol reagent (Gibco/BRL, Grand Island, NY) and quantified. Seven pairs of mRNA samples were hybridized to 14 glass slide arrays prepared from mouse UniGenes at Lawrence Berkeley National Laboratory (Berkeley, CA). RNA from 7 obese mice was paired with RNA from 12 lean mice (5 pools each of 2 lean mice plus 2 lean mice used without any pooling) to produce enough RNA. We minimized pooling of the RNA to allow for individual mouse variability. RNA quality and integrity were confirmed by 1% denaturing gel electrophoresis.

To generate cDNA probes, 50 μg of total RNA was used. RNA and labeling mix were incubated at 70 °C for 10 min, cooled in ice, and added to a prelabeling mix of dNTP (Pharmacia, Bridgewater, NJ), Cy3 or Cy5 dUTP (Amersham Biosciences, Piscataway, NJ), Superscript buffer, and dichlorodiphenyltrichloroethane (Gibco/BRL). After addition of Superscript II (Gibco/BRL), the reverse transcription (RT) was carried out for 90 min at 42 °C. On further

addition of Superscript II, samples were incubated for 90 min at 42 °C, followed by 10 min at 70 °C. After the removal of the unincorporated dyes, using mouse Cot1 DNA (Gibco/BRL), the cDNA probes were hybridized to microarrays. The arrays contained 11,000 expressed sequence tags (ESTs) from IMAGE consortium clones (14). Polymerase chain reaction (PCR)-amplified cDNA inserts were spotted onto poly-L-lysine-coated microscope slides by capillary action using stainless steel pins. The array slides were incubated overnight at 65 °C inside a hybridization oven. The next day, the hybridized slides were washed in 1× standard sodium citrate (SSC) and 0.05% SDS, followed by two additional rinses of 0.2× SSC and 0.1× SSC, respectively. Arrays were then spun dry.

Microarray Scan. Hybridized slides were scanned with a purpose-built dual laser scanner. The data from the acquired images of expressed genes were then analyzed using the ScanAlyze program (http://rana.lbl.gov/EisenSoftware.htm). Grid coordination was used to match the average pixel intensity with its spot identity.

Microarray Statistical Analysis. Statistical analysis was carried out as described (15). In brief, for each experiment,

A) Microarray Experiment Design
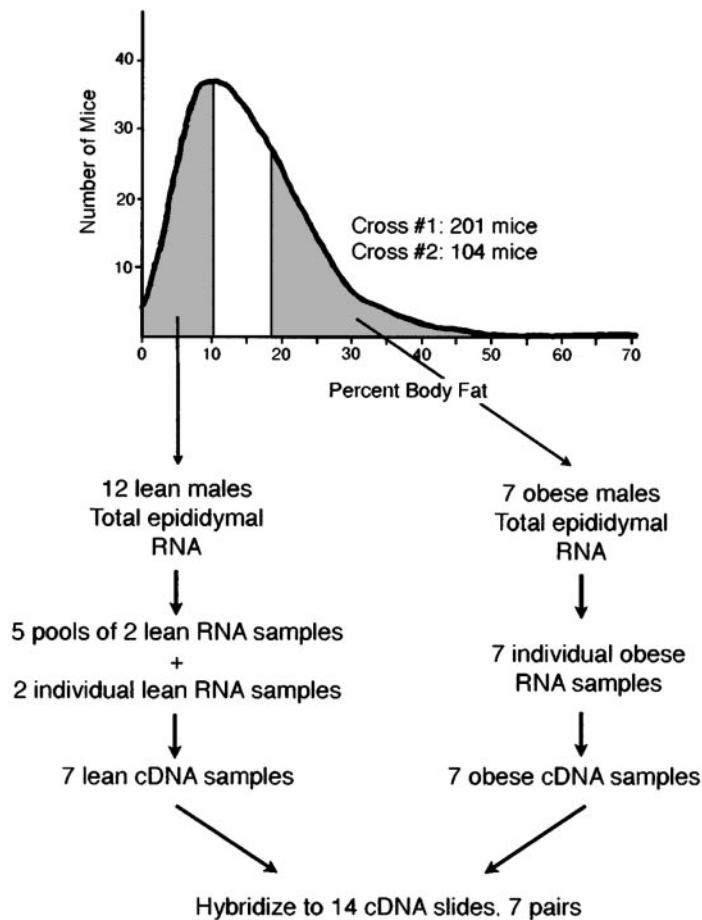
B) Quantitative PCR Experiment Design
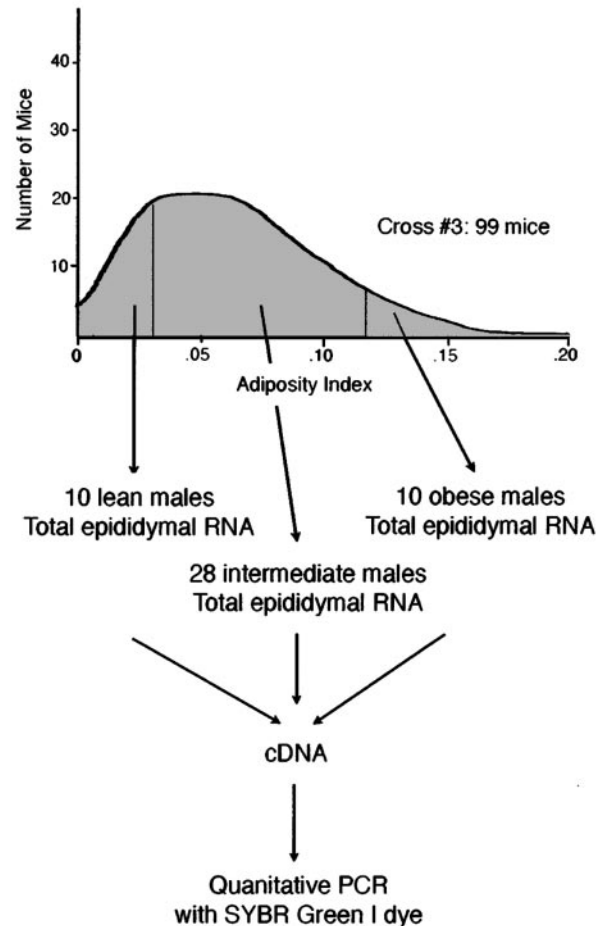
Figure 2: Selection protocols for microarray and quantitative PCR analysis. (A) Adipose from lean and obese mice as defined by percentage body fat were selected for microarray from crosses 1 and 2. Seven pairs of cDNA samples from lean and obese mice were hybridized to 14 microarrays using a dye swap (lean-labeled cy3 with obese-labeled cy5 and lean-labeled cy5 with obese-labeled cy3). (B) Lean and obese mice for quantitative PCR were selected from cross 3.

the expression log ratios were displayed in a two-dimensional matrix with rows corresponding to array elements and columns corresponding to arrays. To test the null hypothesis H_j of equal mean expression for array element j in the lean and obese mice, a two-sample Student's *t* statistic was used. Assessing the strength of the evidence against the null hypotheses of equal expression in the lean and obese mice is typically done by calculating *p* values for each hypothesis. To address the concern of multiple testing, we computed adjusted *p* values for each gene (16). We adopted conservative criteria for reporting the results, requiring both that clones exhibit significant differences for the comparison of lean vs. obese (unadjusted *p* value set arbitrarily at $p \leq 0.001$) and that expression be at least 2-fold differential on the microarray.

Gene Identification and Position. Accession numbers from differentially expressed genes provided the means to obtain the nucleotide sequences from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequences or text were then entered into the nucleotide Basic Local Alignment Search Tool (BLAST) and translated Basic Local Alignment Search Tool query (tBLAST) against the Mouse Ensembl (http://www.ensembl.org/Mus_musculus). The resultant gene calls were screened for identity, with a maximal Expect value threshold of 10^{-4}. In all cases for the positional candidates that mapped to known BSB obesity QTLs, Expect values were below 10^{-15}, with a minimal sequence identity of 85% direct base pair lineup. The Expect value is a parameter that describes the number of hits expected by chance. Chromosomal positions (in base pairs from the Mouse Ensembl) were obtained using sequence and name-based searches. We identified matches of the EST sequences to transcripts and to chromosomal locations in our differentially expressed

Table 1. Phenotypes of mice used for microarray and for quantitative RT-PCR assays

	Age (days)	Body weight (g)	Total fat pad (g)	BMI	Body fat (%)
Mice used for microarray*					
Obese (mean $\pm$ SD), $N = 7$	139 ± 13	44.6 ± 4.1	5.72 ± 1.50	0.38 ± 0.02	27.7 ± 6.0
Range	125 to 159	41.1 to 46.9	4.32 to 8.78	0.35 to 0.41	18.3 to 37.0
Lean† (mean $\pm$ SD), $N = 7$	155 ± 16	22.7 ± 2.3	0.51 ± 0.10	0.24 ± 0.02	7.6 ± 1.3
Range	135 to 176	20.4 to 27.6	0.35 to 0.62	0.22 to 0.26	6.0 to 10.2
p value lean vs. obese	NS	<0.0001	<0.0001	<0.0001	<0.0001
Mice used for quantitative RT-PCR analysis‡					
Obese (mean $\pm$ SD), $N = 10$	204 ± 9	53.2 ± 4.7	7.73 ± 1.96	0.42 ± 0.04	14.4 ± 2.5
Range	194 to 226	45.2 to 61.8	5.82 to 11.80	0.37 to 0.49	11.8 to 19.1
Lean (mean $\pm$ SD), $N = 10$	201 ± 8	25.1 ± 4.9	0.68 ± 0.21	0.24 ± 0.02	2.7 ± 0.8
Range	188 to 211	16.5 to 32.7	0.13 to 0.84	0.19 to 0.27	0.8 to 3.7
p value lean vs. obese	NS	<0.0001	<0.0001	<0.0001	<0.0001
Entire distribution (mean $\pm$ SD), $N = 48$	202 ± 10	36.4 ± 11.2	3.42 ± 2.70	0.320 ± 0.068	8.3 ± 4.4
Range	183 to 226	16.5 to 61.8	0.13 to 11.80	0.187 to 0.493	0.8 to 19.1

* Selected as most obese (all were $Lipc^{S/-}$ genotype) or leanest (all were $Lipc^{B/-}$ genotype) from 305 male BSB mice from $Lipc$ crosses 1 and 2 in Figure 2A.

† Ten lean mice were pooled into five samples prior to analysis.

‡ Twenty of 99 male BSB mice were selected from $Lipc$ cross 3 in Figure 2B and categorized as lean or obese. An additional 28 mice were selected to reflect the entire distribution of obesity.

NS, not significant, $p > 0.05$.

collection by searching through three databases: Mouse Ensembl, Celera mouse genome assembly, and Celera mouse transcript assembly (both available at http://www.celeradiscoverysystem.com). Using these three sources allowed us to obtain robust information on the identity of the differentially expressed genes.

Quantitative Real-Time (qRT)-PCR Analysis

We generated cDNA from 1 μg of total RNA (Applied Biosystems, Foster City, CA) from epididymal adipose tissues from 48 of 99 male mice in cross 3 selected to be approximately equally distributed across a wide range of obesity as measured by AI (Figure 2B; Table 1). qRT-PCR reactions were carried out by using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). We performed the reactions with 1$\times$ SYBR Green I Dye PCR master mix buffer (Applied Biosystems), 30 ng of cDNA as template, and 300 nM of specific primer sets. Primer pairs specific to the validated genes of interest were designed with ABI Primer Express software (Applied Biosystems). Specificity of each primer pair in each sample was validated by melting curve analysis after each PCR using built-in capabilities of the 7900. These analyses confirm the presence of a single product for each primer in each sample.

Samples were run in triplicate reactions to confirm consistency in the amount of PCR products. Amounts of input RNAs in each sample were corrected for by dividing threshold cycle (Ct) of each specific gene by the Ct for calmodulin control. Fold values were calculated as $2^{-\Delta Ct}$, where $\Delta Ct =$ Ct for specific gene in mRNA $-$ Ct for calmodulin in the same RNA. The sample with the lowest expression was set to 1.0-fold, and other data were adjusted to that baseline.

Measurement of Liver Iron, Zinc, and Copper Levels and Plasma Leptin

Liver samples were wet ashed in 16 M HNO_3 for 7 days (17). Samples were then diluted with appropriate amounts of 0.1 M HNO_3 and analyzed for iron, zinc, and copper by atomic absorption spectrophotometry (Model Smith-Heifjie 4000, Thermo Jarrell, Ash, Franklin, MA). Plasma leptin was measured as described previously (18).

Statistical Analysis

Summary data are reported as mean $\pm$ SD. Data were analyzed by ANOVA and regression analysis (StatView 5.0.1; Abacus Concepts Inc., Berkeley, CA). Fold expression values, obesity phenotypes, with the exception of AI, and plasma copper were log transformed before analysis.

Results

cDNA Microarrays

Genes differentially expressed between lean and obese BSB epididymal adipose tissue were identified by hybridization of paired RNAs to cDNA microarrays. Comparison of the expression profiles from the BSB array showed up-regulation of 8 genes and down-regulation of 42 genes among the most obese relative to lean mice, for a total of 50 differentially regulated transcripts (Table 2). Sequence searches of EST sequences from these 50 clones identified chromosomal locations for 45 of the 50 genes. Six differentially expressed EST sequences had well-defined chromosomal positions, but the genes are completely unknown. Only one gene (accession no. AA274549) did not match at all to either Ensembl or Celera databases. Leptin (*Lep*) and adipsin (*Adn*) displayed the greatest fold expression differences. Four of the 50 differentially expressed genes are involved in iron homeostasis: *Hfe*, diaphorase 1, transferrin receptor 2 (*Trfr2*), and protoporphyrinogen oxidase. Five EST sequences were mapped under three obesity QTLs in BSB mice (7) (Table 2), but none of the iron homeostasis genes mapped under the QTLs.

qRT-PCR

We selected six transcripts, *Lep*, cytochrome P450 2e1 (*Cyp2e1*), *Adn*, protein tyrosine phosphatase-like B (*Ptplb*), *Hfe*, and matrix metalloproteinase-11 (*Mmp11*), from our list of differentially regulated transcripts in Table 2 for further validation by qRT-PCR in 10 obese vs. 10 lean mice from BSB cross 3. All of these transcripts confirmed statistically significant ($p \leq 0.05$ and $\geq$2-fold) varied expression when obese mice were compared with lean mice. In addition, we examined expression of three known obesity genes as negative controls for differential expression: glucose transporter 2, uncoupling protein 2, and peroxisome proliferative activated receptor gamma 2. These genes were present on the array but did not meet the criteria of statistical significance and fold change of this study. None of the three obesity genes reached statistical significance with qRT-PCR. We also selected three genes present on the array, molybdenum cofactor synthesis protein 3, four and a half LIM domains 1, and transcriptional enhancer factor-1, that appeared to exhibit differential regulation by an alternate method of analysis (19). Again, none of these reached statistical significance with qRT-PCR. Thus, the microarray as analyzed provided a very efficient method to identify genes likely to be differentially expressed between lean and obese BSB mice.

We then examined expression of 5 of these genes (*Lep*, *Cyp2e1*, *Ptplb*, *Hfe*, and *Mmp11*) in 48 male BSB mice selected to cover a range of obesity (Figure 2B; Table 1). To determine if there was a predictive relationship between fold gene expression and obesity phenotypes, phenotypes were regressed against fold over minimum mRNA expression for these genes (Table 3; Figure 3). *Cyp2e1* and *Mmp11* did not show significant regression on obesity traits. *Lep* expression was significantly associated with all obesity phenotypes except the retroperitoneal fat depot (Table 3), and there was a direct correlation between *Lep* expression and AI (Figure 3A). On the other hand, *Ptplb* was inversely related to body weight, BMI, the weight of the femoral fat pad and the total fat pad mass (Table 3), and AI (Figure 3B). The inverse relationship between *Hfe* and all obesity phenotypes was striking, whereas *Trfr* was associated only with AI (Table 3; Figure 3, C and D).

Expressions of *Lep*, *Ptplb*, and *Hfe* were regressed against age, anal-to-nasal length, body weight, BMI, AI, and the mass of each of the fat depots in a step-wise multivariate analysis to determine which phenotypes in combination were significantly associated with the variance in gene expression in this model. Variation in epididymal adipose depot weight plus body weight (*Hfe*) or BMI (*Lep*) explained 49% and 40%, respectively, of the variance in fold differences in expression of *Hfe* and *Lep*. Variance in *Ptplb* was not significantly explained by the obesity phenotypes.

Iron Homeostasis and Obesity

Table 2 includes 4 genes that are potentially involved in iron homeostasis of just 50 that are differentially expressed between lean and obese mice. Because adipose *Hfe* mRNA is strongly correlated with obesity, we examined the possibility that iron homeostasis is generally correlated with obesity in inbred SPRET/Ei BSB mice. In an attempt to confirm the correlation between adiposity and *Hfe*, we obtained an antibody to human *HFE* and tested it against extracts from adipose tissue of wild-type and *Hfe* knockout mice; unfortunately, the antibody reacted with a protein of the same size in all mice. Thus, we cannot report successful measurement of *Hfe* protein nor its association with obesity. However, we note that no Western blots of mouse hemochromatosis protein in knockout mice have been published. Liver iron levels had a mean of 2323 $\pm$ 845 nmol/g (SD) $n = 40$. These levels are inversely associated with BMI and the mass of the epididymal, retroperitoneal, and femoral fat depots (Table 3) and with AI (Figure 3E).

Next, we searched the microarray data for other genes of iron transport and homeostasis to determine if there was any evidence that they are also differentially expressed even if they did not meet the original stringent requirement for difference between lean and obese. We observed that lactotransferrin, which functions to protect tissues against effects of iron overload, was present on the microarray and had a obese/lean differential expression of 1.6-fold and a p value for lean vs. obese of 0.004. Thus, lactotransferrin expression falls just below the thresholds for both fold difference and p value. We also found that *Trfr* was present

Table 2. Genes differentiating obese from lean mice identified by microarray

Gene name and symbol	GenBank accession number	Chromosome, position (Mb)	Putative gene function	Fold change	p Value
Genes up-regulated in obese mice (obese/lean)					
Semaphorin 4C (Sema4c)	AA048101	1, 37.0	Cell growth/maintenance Hormone receptor/signal transduction Neuronal	2.43	6.9E-04
60S Ribosomal protein L35 (Rpl35)	W85557	2, 39.7	Cell growth/maintenance	2.00	3.6E-05
S100 Calcium-binding protein (S100)	AA230451	3, 91.6	Protein binding	2.45	3.5E-04
Leptin (Lep)*	AA510287	6, 29.0	Macronutrient/energy metabolism Hormone/signal transduction	13.27	3.7E-06
Procollagen, type VI, alpha (Col6a)	W33786	10, 77.1	Cell growth/maintenance Cellular cytoskeleton	2.06	6.5E-04
Hemochromatosis (Hfe)	W11889	13, 23.1	Iron transport/metabolism Immune response	4.25	6.0E-04
Diaphorase 1 (NADH) (Dia1)	AA268398	15, 83.4	Macronutrient/energy metabolism Iron transport/metabolism	2.21	8.7E-04
TBP-associated factor 172 (Btaf1)†	AA386522	19, 36.3	Transcription factor	4.32	4.6E-04
Genes down-regulated in obese mice (lean/obese)					
Protoporphyrinogen oxidase (Ppox)	AA036282	1, 172.0	Iron transport/metabolism	2.59	3.2E-05
Phytanoyl-CoA alpha-hydroxylase (Phyh)	W82212	2, 4.9	Macronutrient/energy metabolism Neuronal	2.4	4.3E-05
Unknown	AA209594	2, 72		3.06	2.22E-05
Unknown	AA290015	2, 102.7		2.09	1.5E-04
Unknown*	AA498640	2, 165.7		3.10	1.5E-05
SNF2/RAD52 related transcription termination factor‡§	AA003731	3, 101.2	Transcription factor	2.61	1.3E-04
Endomucin (Emcn)	AA509765	3, 137.8	Cell growth/maintenance Hormone receptor/signal transduction	2.10	1.9E-04
Transferrin receptor 2 (Trfr2)	AI326398	5, 136.3	Iron transport/metabolism	2.98	7.2E-04
Kruppel-related C2H2 type zinc finger protein (Znf212)†	AA289526	6, 48.1	Transcription factor	2.14	1.0E-04
Caldesmon 1 (Cald1)	AA510479	6, 34.7	Cellular cytoskeleton	2.15	7.6E-04
Predicted gene ICRFP703B1614Q5.6*§	AA404094	7, 99.3		2.57	3.8E-04
cGMP-inhibited 3',5'-cyclic phosphodiesterase B (Pde3b)*	AA185313	7, 102.7	Hormone receptor/signal transduction	3.36	5.0E-04

Table 2. Continued

Gene name and symbol	GenBank accession number	Chromosome, position (Mb)	Putative gene function	Fold change	p Value
Cytochrome P450, 2e1, ethanol inducible (Cyp2e1)*	AI323829	7, 129.8	Macronutrient/energy metabolism	4.52	6.7E-04
Monocytic leukemia zinc finger protein (Runxbp2)†	AA266324	8, 21.6	Cell growth/maintenance, Transcription factor	2.39	9.1E-04
Neuronal membrane glycoprotein M6-A (Gpm6a)	AA269845	8, 54.1	Hormone receptor/signal transduction, Neuronal	2.20	1.4E-04
Matrix metalloproteinase-11 (Mmp11)	W16247	10, 75.7	Cell growth/maintenance	4.95	9.5E-04
Adipsin (Adn)	AI324619	10, 79.4	Immune response, Cell growth/maintenance	15.89	1.4E-05
Heat shock protein, 60 kD (Hspd1)	AA277477	11, 41.2	Cell growth/maintenance, Signal transduction	3.96	4.9E-04
Unknown	AA267699	11, 73.5		3.26	2.3E-04
Protein kinase A related anchor protein (Akap5)†	AI326258	12, 71.0	Hormone receptor/signal transduction	2.27	5.3E-04
Ectonucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2)	AA177363	15, 55.0	Transcription factor, Hormone receptor/signal transduction	3.10	2.1E-04
NMDA receptor glutamate-binding protein (Grina)	AA273669	15, 76.5	Hormone receptor/signal transduction, Neuronal	2.27	7.2E-04
Orphan nuclear hormone receptor (Nr4a1)	AA209882	15, 101.7	Transcription factor, Hormone receptor/signal transduction	2.62	4.2E-04
Protein tyrosine phosphatase-like B (Ptplb)	AA145262	16, 34.8	Cell growth and differentiation	3.74	2.4E-06
Unknown	AI893018	16, 45.6		2.53	7.8E-04
Potassium inwardly rectifying channel, subfamily J, member 15 (Kcnj15)	AA472164	16, 96.2	Cellular cytoskeleton	2.10	5.4E-04
Histocompatibility 2, class II antigen E beta (H2ebI)	AA174620	17, 33.4	Immune response	2.16	2.2E-04
Unknown	AA185346	17, 71.9		2.37	4.8E-04
Protein phosphatase 2C beta isoform (Ppm1b)	AA395957	17, 83.6	Cell growth/maintenance	2.64	2.6E-04
Cullin 2 (Cul2)	AA26633	18, 3.4	Cell growth/maintenance, Transcription factor	2.68	1.0E-03
Transcription factor (Bteb1)	AA124387	19, 22.4	Cell growth/maintenance, Transcription factor	3.34	2.0E-04

Table 2. Continued

Gene name and symbol	GenBank accession number	Chromosome, position (Mb)	Putative gene function	Fold change	p Value
3′-Phosphoadenosine 5′-phosphosulfate synthase 2 (*Papss2*)	AA244536	19, 32.1	Cell growth/maintenance	3.99	3.8E-05
Glycerol-3-phosphate acyltransferase, mitochondrial (*Gpat*)	AA209041	19, 54.8	Macronutrient/energy metabolism	2.88	4.8E-04
Interleukin 1 receptor-associated kinase (*Irak1*)	AA590782	X, 58.1	Cell growth/maintenance Hormone receptor/signal transduction	2.90	9.5E-04
UDP-*N*-acetylglucosaminyltransferase (*Ogt*)†	AA119676	X, 86.5	Immune response Macronutrient/energy metabolism	2.81	1.2E-04
Claudin 2 (*Cldn2*)	AA498630	X, 121.6	Signal transduction Cellular cytoskeleton	2.98	6.1E-05
Pyruvate dehydrogenase E1 alpha subunit (*Pdha1*)	AA466268	X, 140.1	Macronutrient/energy metabolism	2.86	9.8E-04
Unknown	AA274549			2.18	6.6E-04
Unknown¶	W97158			2.16	2.8E-04
Unknown¶	AA254115			2.74	3.3E-04
Unknown¶	AA175346			4.40	8.6E-05
Unknown¶	AA122640			3.25	1.0E-03

* Mapped to BSB obesity QTL regions.
† Named by homology in Ensembl.
¶ Identification ambiguous in Celera and Ensembl assembly.
‡ Not identified by name or homology in Ensembl.
§ No gene symbol.

Table 3. Regression of fold expression of leptin, protein tyrosine phosphatase-like B, hemochromatosis, and transferrin receptor measured by qRT-PCR and liver iron with obesity phenotypes in BSB mice

Gene Name	Phenotype	r^2	p of regression
Leptin	Body weight	0.21	0.003
	BMI	0.26	0.001
	Epididymal fat pad	0.10	0.05
	Mesenteric fat pad	0.21	0.004
	Femoral fat pad	0.20	0.004
	Total fat pads	0.14	0.02
Protein tyrosine phosphatase like b	Body weight	0.10	0.04
	BMI	0.11	0.03
	Femoral fat pad	0.10	0.04
	Total fat pads	0.09	0.05
Hemochromatosis	Body weight	0.24	0.0008
	BMI	0.31	0.0001
	Epididymal fat pad	0.51	<0.0001
	Mesenteric fat pad	0.20	0.003
	Retroperitoneal fat pad	0.40	<0.0001
	Femoral fat pad	0.32	<0.0001
	Total fat pads	0.45	<0.0001
Liver iron	BMI	0.16	0.01
	Epididymal fat pad	0.14	0.02
	Retroperitoneal fat pad	0.16	0.01
	Femoral fat pad	0.13	0.02
	Total fat pads	0.12	0.03

All data were log transformed prior to analysis.

on the microarray and had a 2.5-fold obese/lean differential expression but with a p value > 0.1 for the comparison of lean vs. obese.

Next, we used qRT-PCR to quantify adipose mRNA for one of these candidate differentially expressed genes, *Trfr*, and for a gene of iron homeostasis that was not present on the microarray, hepcidin, a liver-expressed gene involved in iron homeostasis. There was a suggestive but nonsignificant correlation of liver iron levels and adipose *Trfr* expression, with $r^2 = 0.08$ and $p = 0.087$. There was a particularly strong correlation of liver copper levels and adipose *Trfr* levels (Figure 3F). *Trfr* and *Hfe* mRNA levels from adipose tissue were not correlated ($p > 0.05$). However, there was an inverse association between *Trfr* and AI (Figure 3D). Using multivariate analysis, 37% of the variance in *Trfr* expression was predicted by a combination of AI, weight of the femoral fat pad, and age. Hepcidin mRNA levels were barely detectable by real-time PCR in adipose tissue and were also not correlated with *Hfe*, *Trfr*, or any other phenotype measured in these BSB mice. These results are consistent with previous observations that hepcidin expres-

sion is liver specific. None of these genes involved in iron homeostasis is located under BSB QTLs; therefore, none is a positional candidate for the BSB obesity model.

Are Alleles of the **Lep** *Gene Positional Candidate Obesity Genes in BSB Mice?*

We previously found that BSB mice produced from a cross involving outbred SPRET/Pt mice have an obesity QTL on chromosome 6 that includes the *Lep* gene within the peak (7). Because *Lep* mRNA levels from BSB mice made with inbred SPRET/Ei are highly correlated with obesity, we examined the evidence that *Lep* is a positional candidate in inbred BSB mice.

Plasma leptin was measured in 327 SPRET/Pt BSB mice. We observed highly significant linkage of microsatellite markers near *Lep* to plasma leptin levels. Maximum logarithm of odds score determined by Mapmanager was a suggestive 1.9 (data not shown), whereas the p values for marker *D6Mit1* (7.7-cM distance from *Lep*) was 0.003 and for marker *D6Mit8* (24.7 cM from *Lep*) was 0.008. Although these results would not be significant for a whole-
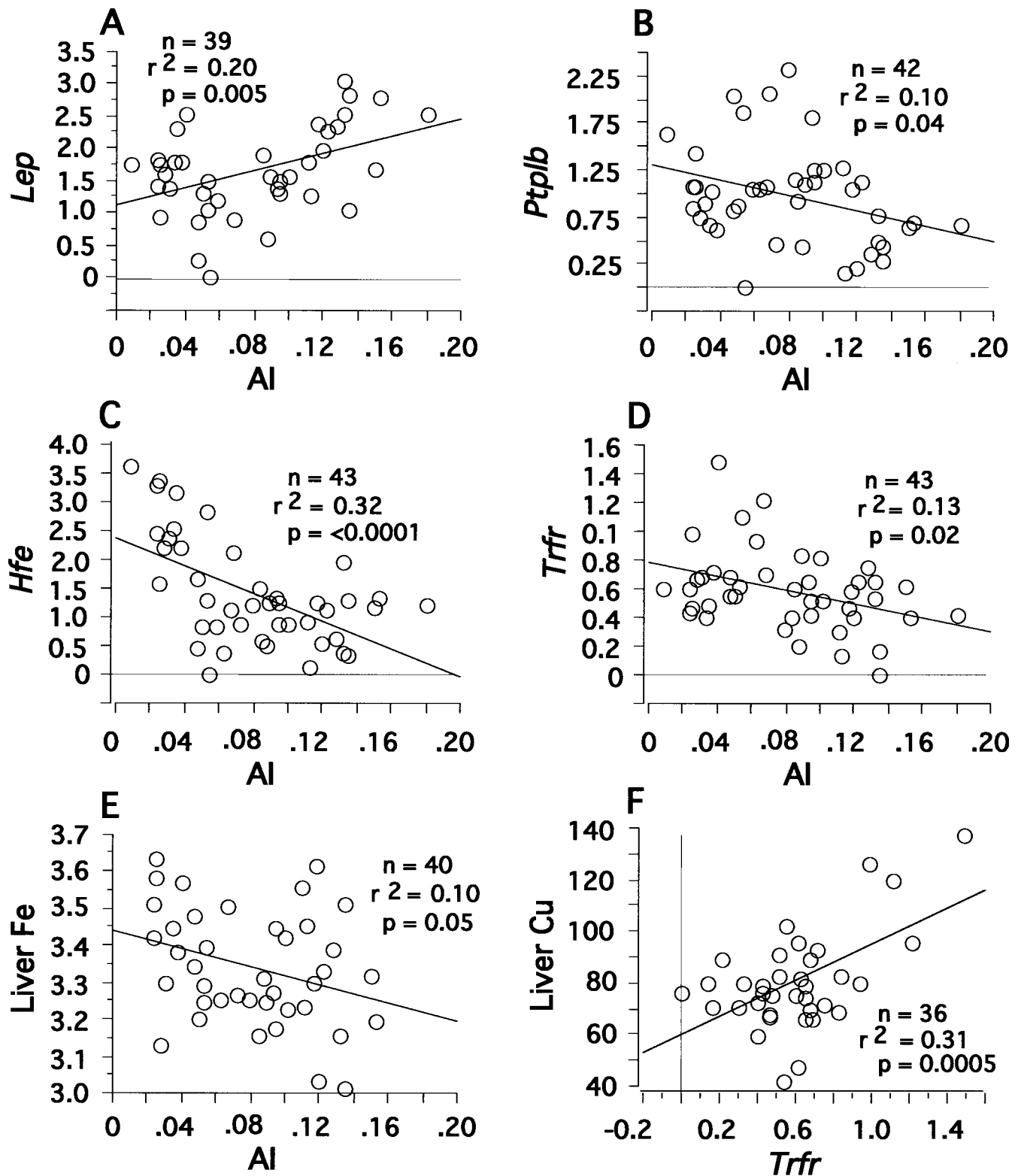
Figure 3: Regression of *Lep*, *Ptplb*, *Hfe*, and *Trfr* epididymal adipose tissue mRNA expression against AI in BSB mice from cross 3. mRNA levels were measured by real-time PCR and expressed as log (fold over minimum). Also shown are correlations of liver iron levels with AI and liver copper levels with adipose expression of *Trfr*.
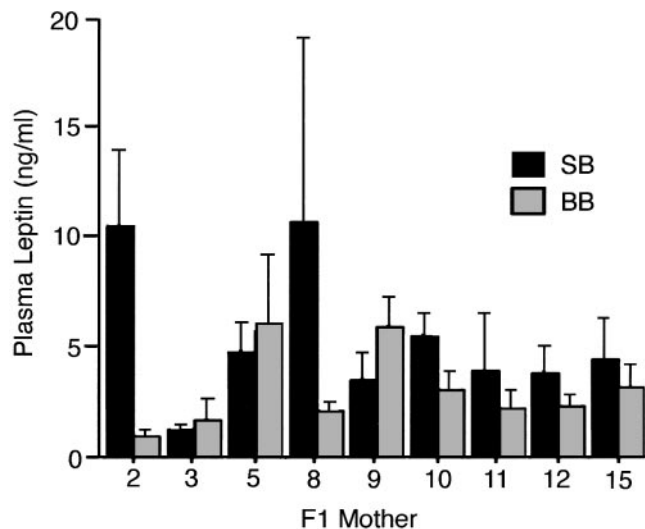
Figure 4: Plasma leptin levels in progeny from outbred SPRET/Pt BSB F1 mothers, split by genotype at *D6Mit1*. Plasma leptin levels were measured in progeny from individual F1 mothers having more than two young from the cross using outbred SPRET/Pt mice. Progeny were genotyped for *D6Mit1*, which is 7.7 cM from the *Lep* gene. More than 92% of mice with SB genotype inherited a *spretus* allele of the *Lep* gene from their mother, whereas <8% had a recombination that resulted in a B6 *Lep* allele being linked to *spretus* at *D6Mit1*. Leptin differed between SB progeny of mothers 2 and 3 ($p = 0.005$) and between mothers 8 and 3 ($p = 0.02$).

genome scan, they were highly significant for a specific hypothesis test that *Lep* gene alleles influence plasma leptin. However, each F1 mother used to produce BSB mice from SPRET/Pt may have had different haplotypes that could have been detected by testing for significant differences among the progeny. We found that there was a >10-fold range of plasma leptin values for progeny with "SB" genotype at *D6Mit1* that were derived from different F1 mothers (highest vs. lowest F1 mothers, $p = 0.009$). Figure 4 shows that the *D6Mit1* genotype effect on leptin was derived primarily from the SPRET/Pt alleles producing high leptin in the progeny of just 2 of the F1 mothers. In contrast, there were no significant differences between the BB genotype progeny of any F1 mother.

Next, we examined linkage of the *Lep* alleles to *Lep* mRNA, plasma leptin, and obesity in BSB mice produced from inbred SPRET/Ei. We observed that there was no significant linkage of marker *D6Mit309* (5.2 cM from *Lep*) to *Lep* mRNA levels (data not shown). We also measured plasma leptin in 105 mice from cross 1 and found no linkage to the *Lep* locus (data not shown). There was no linkage of *D6Mit1* to obesity in BSB mice produced from inbred SPRET/Ei (data not shown). Finally, we tested for significant effects on progeny of F1 mothers from the SPRET/Ei cross. There were no significant differences between progeny from any of the inbred SPRET/Ei mothers.

Discussion

Our central findings are that liver iron levels and genes for iron transport are correlated with obesity in the BSB model and that alleles of *Lep* are positional candidates in BSBs derived from some SPRET strains but not others.

There are coordinate alterations of liver iron and copper levels and six genes for iron transport and homeostasis in adipose tissue in BSB mice: *Hfe*, *Trfr1* and *Trfr2*, lactotransferrin, diaphorase 1, and protoporphyrinogen oxidase. None of these genes is located under BSB QTLs; therefore, these correlations are secondary to obesity in BSB mice. Alternatively, these genes of iron homeostasis could be coordinately regulated by an unknown BSB positional candidate gene.

The influence of the *ob* mutation on iron metabolism was examined in two studies (20,21). Obese (*ob/ob*) mice fed ad libitum absorbed more iron from an oral load, had higher blood hemoglobin and hematocrit, and more iron in their adipose tissue but less in their liver than lean controls (21). Similarly, the concentration of iron in muscle was greater in obese Zucker (*fa/fa*) rats than in lean controls (22). The altered absorption and concentrations of iron in these obese models may reflect an adaptation to supply increased requirements for the expanded blood volume in obese animals (21). A reduction in liver iron stores in association with obesity in BSB mice is consistent with these studies. In humans, mutation of *HFE* results in increased serum iron, transferrin saturation, and ferritin. Three human studies, two population studies (23,24) and the other a twin study (25), showed that obesity per se had highly significant effects on iron indices. In *HFE* wild-type subjects, obesity increased ferritin and decreased serum iron and transferrin saturation (24). In a cross section of Mexican-American men from the Third National Health and Nutrition Examination Survey, serum ferritin concentration was associated with waist-to-hip ratio and other indices of fat distribution and obesity (23). We cannot directly compare our results demonstrating correlation of liver iron and obesity in BSB mice with results from human studies because there have been no studies of liver iron in healthy obese humans.

Our study is the first to show, to our knowledge, correlation with obesity of mRNA levels for genes of iron homeostasis. In addition, ours is the first demonstration, to our knowledge, that *Hfe* mRNA is regulated in adipose tissue, suggesting that it may be regulated in any tissue in any species. Finally, although previous work based on studies of primary adipocytes and adipocyte cell lines suggested that *Trfr* mRNA levels can be used as a control gene for quantitative PCR (26), our studies demonstrate that *Trfr* mRNA levels are regulated in vivo by obesity. These results suggest a cautionary note for gene expression studies because measurement of mRNA levels in primary adipocytes apparently does not reflect in vivo regulation for *Trfr*.

One of the unique advantages of using the BSB model, rather than inbred strains, for studies of differentially ex-

pressed genes is the ability to use regression analysis to explore more fully the relationships of quantitative mRNA expression with quantitative obesity traits. Of the six transcripts examined, four showed significant regression against obesity phenotypes. The most surprising of these is adipose tissue mRNA levels for *Hfe* that are highly correlated with obesity phenotypes. *Hfe* binds to the transferrin receptor, reducing its affinity for iron-loaded transferrin (27), and, as a consequence, reduces cellular iron by reducing iron uptake. The additional correlation of *Trfr* and potential correlation of *Trfr2* with obesity are consistent with the hypothesis that obesity in BSB mice is accompanied by coordinate changes in iron homeostasis. In addition, three other genes implicated in iron metabolism were differentially expressed between lean and obese mice. The array studies in other murine models of obesity did not report differential expression of genes involved in iron metabolism (4,5,28).

Alleles of the *Lep* gene are positional candidates for effects on obesity in some SPRET but not in others. These results are most evident in the significant F1 mother effects on progeny phenotypes of crosses involving the outbred SPRET/Pt and not in crosses involving the inbred SPRET/Ei. These results may aid in the positional localization of the gene underlying the obesity QTL in the same way that haplotypes aided isolation of a gene influencing epidermal cancer (11). Although our results do not prove that alleles of *Lep* influence obesity and plasma leptin, they are highly consistent with the hypothesis that alleles of *Lep* from some outbred SPRET have a significantly greater effect on plasma leptin than other leptin alleles. Plasma leptin maps to the BSB QTL on chromosome 6 in the BSB cross from outbred SPRET/Pt but not inbred SPRET/Ei.

Several other differential transcripts also map to BSB obesity QTL regions, including an unknown gene on chromosome 2 and three genes (cyclic guanosine 3′,5′-monophosphate-inhibited 3′,5′-cyclic phosphodiesterase B, *Cyp2e1*, and predicted gene ICRFP703B1614Q5.6) on chromosome 7. Alleles of these genes may be underlying causes of the QTLs, their differential regulation may be caused by allelic effects that are unrelated to obesity, or they may be differentially regulated by adiposity.

Our results demonstrate that cDNA microarrays provide only an approximate guide toward identification of differentially expressed genes because they produced results with inverted correlations between *Hfe* and *Cyp2e1* and obesity relative to results from RT-PCR. cDNA microarray analyses have many known limitations, including cross-hybridization of cDNA from related genes. Our results reemphasize the need to confirm cDNA microarray results with an independent method, such as real-time PCR.

We identified 50 genes differentially expressed in epididymal adipose tissue between lean and obese BSB mice by using a microarray with 11,000 cDNA clones using conservative criteria for differential expression. We identified 124 differentially expressed genes with a *p* value of ≤0.001 and found 1265 genes of 11,000 with expressions that were altered by ≥2-fold, lean vs. obese. Thus, the 50 genes reported here were selected to minimize false positives and maximize differential expression. Although other analytical methods would indicate more genes and pathways with positive results, we focused on these few novel positive results because they concentrate attention on these few genes most differentially expressed. We validated our array results by qRT-PCR from an independent sample of lean and obese BSB mice for 6 of the 50 differentially expressed genes on the array. Differential transcripts were confirmed for all of the six genes when comparing only the very lean with the most obese. However, only three of the six transcripts showed significant regression against some obesity phenotypes across a range of adiposity. Of the six transcripts, *Lep* and *Hfe* had large fold changes, 7 and 50, respectively with qRT-PCR. For the remaining transcripts with lower fold differences, measurement error may have been so large as to obscure a relationship between fold expression and obesity phenotypes.

The genes with the highest calculated fold change from the cDNA microarray, *Adn* and *Lep*, play important regulatory roles in obesity (see Table 2 for putative gene functions). *Adn*, a serine protease, is normally down-regulated in rodent adipocytes as a result of elevated glucocorticoids and insulin levels in obese animals (29–31). The adipsin/acylation-stimulating protein pathway regulates triglyceride synthesis in adipose cells (32). Leptin is a well-known adipocyte-derived hormone that is elevated in obesity and plays a major role in food intake, energy expenditure, and body weight regulation through central and peripheral signals (33). *Ptplb* is a member of a protein tyrosine phosphatase family involved in cell growth and differentiation (34). *Mmp11* was also strongly differentially expressed, lean vs. obese, but did not correlate with the full range of obesity. Matrix metalloproteinases, which are essential for obesity-mediated adipose tissue formation, were shown recently to be differentially expressed in adipose tissue during obesity (35).

Overall, our results suggest that iron homeostasis is coordinately regulated in vivo in fat depots in response to an obesity-related alteration of iron homeostasis. Because alterations of iron in various tissues can have broad impacts on the physiological functioning of each tissue, generation of reactive oxygen species and energy metabolism of adipose tissue of the obese may be altered in part by correlated changes in iron homeostasis. We also demonstrated that *Lep* alleles may be positional candidates for obesity or plasma leptin effects in some outbred strains of SPRET.

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